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The objective of the project is to establish and use an *in vitro* system of intact hepatocytes from rodent and human liver to develop quantitative data on the metabolism of toxic chemicals that can be used in risk assessments. The following halogenated aliphatic solvents are being studied: chloroform, 1,1,1-trichloroethane, trichloroethylene, dichloromethane, bromochloromethane, and carbon tetrachloride. During the first year of the project, experiments were performed with hepatocytes isolated from male F344 rats and the precision-cut liver slice method was established for use with rat and human liver. In the hepatocyte experiments, the cells were incubated in gas-tight flasks with chloroform to establish a model for measuring the rate of its disappearance. Using low concentrations of chloroform (2400 pmol per flask and 9350 pmol per flask), it was found that chloroform in the headspace of flasks containing cells rapidly decreased while it remained unchanged in control flasks. Chloroform levels in the medium also decreased with time. Future experiments will more completely characterize the kinetics of chloroform disappearance with isolated hepatocytes and compare them to precision-cut liver slices.

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IN VITRO SYSTEM FOR STUDYING METABOLISM OF ENVIRONMENTAL CHEMICALS IN HUMAN CELLS

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LIST OF PERSONNEL

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Jack E. Dabbs	Task leader: hepatocyte isolations, liver slice preparation, incubations, toxicity measurements
G. Ross Gordon	Gas chromatographic analysis

OBJECTIVES

The objective of this project is to establish and use an *in vitro* system of intact isolated cells from rodent and human tissues to develop quantitative data on the metabolism of toxic chemicals that can be used for risk assessments. The following halogenated aliphatic solvents are being studied: chloroform, 1,1,1-trichloroethane, trichloroethylene, dichloromethane, bromochloromethane, and carbon tetrachloride. In the first phase of the project, these six compounds are being used with isolated rat hepatocytes to establish the conditions for the generation of kinetic constants for metabolism in these cells, the constants being determined as disappearance of the parent compound. Several experimental variables are being modified to optimize the correspondence between the *in vitro* results obtained and published *in vivo* data, including the comparison of isolated hepatocytes to precision-cut liver slices. This work is currently in progress. In the second phase, the system developed with rat liver cells will be applied to mouse and human hepatocyte incubations. The same six halogenated solvents will be studied and the resulting data will allow quantitative comparison of the metabolism and cytotoxicity in the three species. Additional studies will be performed with trichloroethylene or some other model compound in the final phase of the study to generate metabolite profiles at several concentrations. These data will be analyzed to characterize interspecies differences in the kinetics of the production of multiple metabolites.

MATERIALS AND METHODS

Hepatocyte Isolation

Hepatocytes were isolated from male F344 rats (300 - 450 g) by the whole-liver perfusion method (Green et al., 1983). The cell yield and viability were determined by counting the cell suspension using a hemacytometer and calculating the percentage of cells that excluded trypan blue. The cell yield and initial viability for the experiments presented in this report averaged 717 $\times$ 10^6/rat and 90%, respectively.

Isolated hepatocytes were suspended in a modified Waymouth's 752/1 culture medium (CMH) that contained 11.2 μg/ml alanine, 12.8 μg/ml serine, 24.0 μg/ml asparagine, 84.0 μg/ml gentamicin sulfate, 0.168 μg/ml aminolevulinic acid, 5.0 μg/ml oleic acid, 5.0 μg/ml linoleic acid, 1.0 μg/ml D,L-tocopherol, 288 ng/ml testosterone, 272 ng/ml estradiol, 393 ng/ml dexamethasone, 7.9 μg/ml thyroxin, 30 ng/ml glucagon, 0.02 U/ml insulin, and 0.2 % bovine serum albumin (BSA).

Incubation of Hepatocytes with Halogenated Solvent

The hepatocytes were prepared at a density of 1 $\times$ 10^6/ml or 4 $\times$ 10^6/ml, and 4 ml of cell suspension was added to each side-arm incubation flask. The flasks were matched for total volume (about 25 ml) and outfitted with gas-tight valves (Mininert valves, Pierce, Rockford, IL) to fit both the top and side-arm opening of the flaskto allow repeat sampling of both the head space and the medium. After addition of the cells, the flasks were gassed vigorously for about 30 s with 95% air:5% CO_2 and immediately stoppered. The solvent (chloroform in the studies described in this report) was added with a Hamilton syringe through the Mininert valve directly into the medium. The flasks were placed in an oscillating water bath and maintained at 37°C at 60-70 oscillations per minute.

The stock solutions of chloroform were prepared by dissolving the halogenated hydrocarbon in dimethyl sulfoxide (DMSO) and then diluting it into CMH in a sealed vial. The air/medium partition coefficient was assumed to be 0.13 (Tyson et al., 1983). This value was used in determining the amount of chloroform that should be added to the flask to achieve concentrations of about 0.1 and 1.0 μM. Chloroform (27 μl) was mixed with DMSO (4.9 ml). This stock solution was diluted in culture medium, 1:1000 and 1:10,000. Aliquots (0.1 ml) of these solutions were added directly to the media of the flasks as described above.

Samples for analysis were obtained at intervals of 30 min, or in some experiments more frequently. Media samples were removed through the side-arm valve with a Hamilton syringe, and air samples were removed through the top valve with an air-tight syringe. Air samples were injected directly into the gas chromatograph. Media samples (0.1 ml or 0.05 ml) were added to vials containing either methanol (1.8 ml) or saturated urea (0.6 ml) and stored on dry ice until analysis.

Gas Chromatographic Analysis of Chloroform

Chloroform was analyzed using gas chromatography with a 2 mm × 6 ft glass column packed with 0.1% SP1000 on 80/100 Carbopack C. A Varian Model 3700 gas chromatograph equipped with an electron capture detector and a Hewlett-Packard model 3390A integrator was used. The following parameters were used: column temperature, 125°C; detector temperature, 150°C; injector, 250°C, N_2 flow rate, 30 ml/min.

A standard curve for chloroform was obtained as follows: chloroform, 10 μl, was added to DMSO, 4.9 ml, in a 1-dram vial with a Teflon septum. An aliquot (10 μl) was further diluted with 1.99 ml of methanol. Standard solutions for injection were prepared by diluting 20, 60, 125, and 200 μl to a volume of 2 ml with methanol in septum-sealed 0.5-dram vials to yield 0.15 to 1.5 ng of chloroform per microliter. These solutions were injected directly into the gas chromatograph. The linearity and sensitivity of the standard curve is shown in Figure 1.

Media aliquots (0.05 ml) were removed from the closed flasks through a Mininert valve and added to a 1-dram vial (15 × 45 mm, 4.9 ml) through a Teflon-lined septum. The vial contained 0.6 ml of saturated urea and was stored on dry ice prior to and after the addition of the sample aliquot. Chloroform was released into the vial headspace when the vial was allowed to thaw at room temperature and then heated to 60°C for 10 min. The headspace of the vial was then sampled (0.5 or 1.0 ml) with a gas-tight syringe and the sample injected in the gas chromatograph.

Lactate Dehydrogenase Measurements

At the final time point in each experiment, two aliquots of medium (250 μl each) were removed from each incubation flask. One aliquot was mixed with an equal volume of 0.1% Triton X100 and allowed to sit for about 10 min. This sample was used to measure total lactate dehydrogenase (LDH)--cellular plus released enzyme. The other aliquot was centrifuged at about 2500 × *g* for 5 min to pellet the cells and the supernatant removed for later determination of released LDH. These samples were stored at 4°C for 24-48 h and then assayed using a semi-automated method (Gemeni Mini-centrifugal Analyzer) based on the measurement of the changes in NADH (Tyson

and Green, 1987). The results were calculated as percentage of total LDH released to the medium.

STATUS OF RESEARCH EFFORT

Previous studies conducted by SRI established the dose-response relationship for cytotoxicity of several chlorinated solvents to rat hepatocytes (Tyson et al., 1983) by using relatively high concentrations and short exposure periods. For example, in these studies, the EC50 (concentration at which 50% of the cellular LDH was released to the medium in 2 h) for chloroform was calculated to be 7.1 ± 0.49 mM. At these concentrations, a decrease in chlorinated hydrocarbon level in the medium was not detected. The metabolite profile of another chlorinated hydrocarbon, trichloroethylene, was well-characterized by our laboratory using rat and human hepatocytes (Knadle et al., 1990); the purpose of this study was to compare the disposition of this solvent in the two species and the formation of particular metabolites as a means for predicting hepatocarcinogenic risk to humans. In that study, hepatocytes from both species metabolized trichloroethylene to trichloroethanol and its glucuronide, chloral hydrate, and trichloroacetic acid. The rate and extent of metabolism was greater in rat than human hepatocytes. The pattern of metabolites also varied with species. Rat hepatocytes formed proportionally more trichloroacetic acid, the metabolite believed to be responsible for the hepatocarcinogenicity of trichloroethylene (Elcombe, 1985).

The purpose of the current experiments is to extend this earlier work and establish an *in vitro* technique for readily determining kinetic constants for metabolism of chlorinated solvents. Since the literature suggests that the Km values for the chlorinated solvents that we plan to study are in the micromolar range (Gargas et al., 1986), it was necessary to establish new techniques to allow the investigation of much lower concentrations than used previously.

During the course of these experiments, new information led us to consider whether the basic *in vitro* model for these studies should be modified. We have observed in other experiments that the viability of human hepatocytes in suspension culture decreases much faster than that of rat liver cells. On the average, rat hepatocytes leak about 5-10% of the total LDH into the extracellular medium during a 4-h incubation period. Human hepatocytes lose at least 20% of the total LDH to the medium---and some preparations release as much as 60-80%---in 4 h. This observation led us to conduct a study that compared the metabolism of a test compound (currently client private information) in suspension and monolayer cultures of human hepatocytes. In that study, human hepatocyte monolayer cultures were found to metabolize the drug with a higher V_{max} (similar to that obtained with human liver microsomes) and form a more complete metabolite profile than human hepatocyte suspension cultures (Green et al., manuscript in preparation). In contrast, no difference was found in the rate of benzo(a)pyrene metabolism by rat hepatocytes in suspension and monolayer cultures (Knadle et al., manuscript in preparation). Unfortunately, monolayer cultures are

probably not a practical alternative system for the present studies. Volatile compounds are very difficult to handle in monolayer cultures because the halogenated solvents dissolve into the plastic of the culture dishes and hepatocytes do not attach to glass culture vessels. An additional problem is that human hepatocytes do not attach efficiently in monolayer culture even when plastic dishes and extracellular matrices are used. Therefore, we decided to investigate precision-cut liver slices, an alternative *in vitro* system that has been developed as a model for metabolism studies and reported to be useful with human and rat liver specimens.

Hepatocytes

In the earlier work, we added the volatile test chemicals to a center well in the flask and allowed them to equilibrate between the air and medium phases (Tyson et al., 1983; Knadle et al., 1990). This worked well when microliter quantities were added to each flask. However, for the current experiments we hoped to achieve exposure concentrations less than or equal to 10 μM, or the equivalent of adding less than 1 nl to each flask. Thus, we made serial dilutions in DMSO and then culture medium and added the diluted chloroform directly to the medium. Chloroform equilibrated between the medium and headspace in less than 5 min. The amount of chloroform to be added was estimated by using a published partition coefficient for air and media (0.13; Tyson et al., 1983) to calculate the amount needed to give concentrations in the range of 10, 1, and 0.1 μM. The total amount of chloroform per flask has not been determined in all experiments but is being obtained all current and future experiments.

In initial experiments, we attempted to determine the concentration of chloroform in the medium by using previously used methods (Tyson et al., 1983) in which the medium sample was diluted into ice-cold methanol and this solution analyzed directly by gas chromatography. However, the concentrations of chloroform studied were much lower than those previously used and, as a result, components in the culture media coeluted with the chloroform, interfering with quantitation. Therefore, the technique described in the Methods section was developed, i.e., in which the chloroform is volatilized into the headspace of a sealed vial and analyzed by direct injection of the headspace. This technique was originally developed for measuring volatile solvents in serum (Pryor et al., 1991) and has been applied to toluene, chlorobenzene, xylene, and chloroform. Recovery using this method is essentially 100%.

In experiments conducted with hepatocytes at a density of 1×10^6/ml, no change in chloroform concentration in the media or headspace was detected. Increasing the cell number to 4×10^6/ml resulted in a steady loss of chloroform in the media, from 0.33 μM at 30 min of incubation to 0.12 μM at 240 min. There was no significant change in chloroform concentration from flasks that did not contain hepatocytes during the incubation period.

Chloroform was readily measured in the headspace of the incubation flasks. Experiments with samples taken at 30, 60, 90, and 120 min showed that, at the first time point (30 min), flasks containing hepatocytes and 2400 pmol chloroform had only about 3% as much chloroform in the headspace as control flasks without cells (6.5 nmol and 211 nmol, respectively). Hepatocytes incubated with 9200 pmol chloroform behaved similarly. In this experiment, the headspace chloroform levels continued to decrease with time up to 120 min in flasks containing cells but remained constant in control flasks (Figure 2). The difference between the amount of chloroform in the air in flasks with hepatocytes and that in control flasks suggests that metabolism is actually occurring very early, within the first 30 min of the incubation. The amount of chloroform in the medium confirmed this idea, since in this experiment it remained relatively unchanged between 30 and 120 min in flasks with cells and was lower than the amount of chloroform in control flasks.

Figure 3 shows the results of an experiment in which time points prior to 30 min were analyzed. Chloroform was rapidly lost from the headspace of flasks containing rat hepatocytes. The chloroform concentration in the medium was not determined in this experiment but will be in the future to test our hypothesis that it is also decreasing rapidly under these experimental conditions and to allow determination of the dose of chloroform placed in the flask. The measurement of the headspace concentration may prove to be a reliable technique for determining metabolism in these *in vitro* preparations, analogous to the method that has been used with subcellular fractions (Sato and Nakajima, 1979) and *in vivo* gas uptake studies (Gargas et al., 1986).

LDH release was also measured in these experiments, although cytotoxicity was not anticipatedat the low concentrations studied. However, a small increase in LDH release has been detected in several experiments in hepatocytes treated with chloroform (3% of the total LDH in control cells compared to 8% of the total LDH in treated cells). It seems unlikely that this slight increase is due to the chloroform, which is present in concentrations less than 1 μM in most experiments. Instead it may be an artifact associated with the frequent sampling of treated cells. Aliquots for LDH analysis are removed from untreated hepatocytes only at the beginning and end of the incubation period from the side-arm by removing the Mininert valve. Treated cells are sampled frequently through the Mininert valve to measure medium chloroform levels, and the Hamilton syringe used may lyse cells and thus elevate extracellular LDH levels.

Liver Slices

The work performed to date with precision-cut liver slices has involved acquisition of the necessary equipment and establishing the techniques for preparing and handling the slices in our laboratory. The liver slice system was developed at the University of Arizona, Tucson, in the laboratories of Drs. I. Glenn Sipes and Klaus Brendel, as an alternative to the classical tissue slice preparation methods; the new method resulted in highly reproducible preparations in terms of thickness and maintenance of viability

(Smith et al., 1986). The tissue slices prepared using the Krumdieck tissue slicer (Krumdieck et al., 1980) not only are thinner (approximately 250 μm thick) but are also processed in the presence of a physiological buffer, which facilitates cutting and reduces the trauma associated with slice preparation.

Briefly, a core of liver is prepared from a dissected lobe of the liver (rat or human) by using a sharpened cylindrical stainless tube attached to a drill press. The core is then placed into the cylindrical tissue holder in the Krumdieck slicer. The core is lightly compressed with a piston holding an adjustable weight at constant pressure for slicing evenly throughout the entire length of the core. Slicing is accomplished by pulling the immobilized cylindrical tissue holder across a motor-driven oscillating blade. During the slicing process, the core is emersed in culture medium, pH 7.4, and a gentle stream of buffer diverts the freshly sectioned slices from the blade to a collecting chamber.

The slices are then transferred to a shallow tray containing medium and irregularly shaped ones are discarded. The slices are then loaded onto the screen mesh of round inserts that fit in scintillation vials (three slices per vial). Sufficient medium is added to each vial that the slices move in and out of the medium as the vials rotate on a heated incubator (4 revolutions/min). The slices are preincubated for 90 min to allow recovery from the slice procedure, after which the medium is changed and test chemicals added. We have adapted the Mininert valves to fit scintillation vials so the halogenated hydrocarbon can be added directly to the medium as in the hepatocyte experiments and both headspace and medium can be sampled through the valve. In preliminary experiments, we have found that the viability of the rat liver slices is well maintained during a 240 min incubation period (less than 10% LDH release) and a cytochrome P450 associated activity, 7-ethoxycoumarin O-deethylation, remains constant. Experiments in which the metabolism of chloroform is determined in rat liver slices and hepatocytes from the same animal are planned are planned.

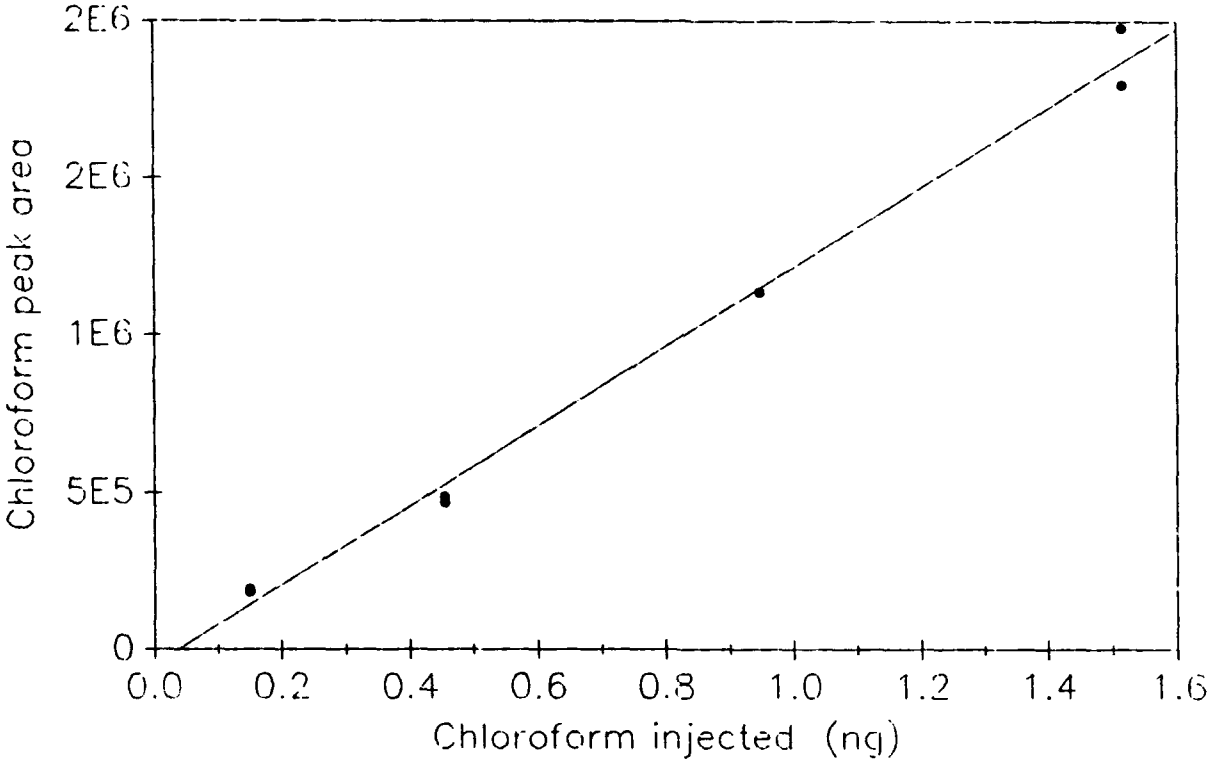

Figure 1. **Standard curve for chloroform obtained using gas chromatographic analysis.**

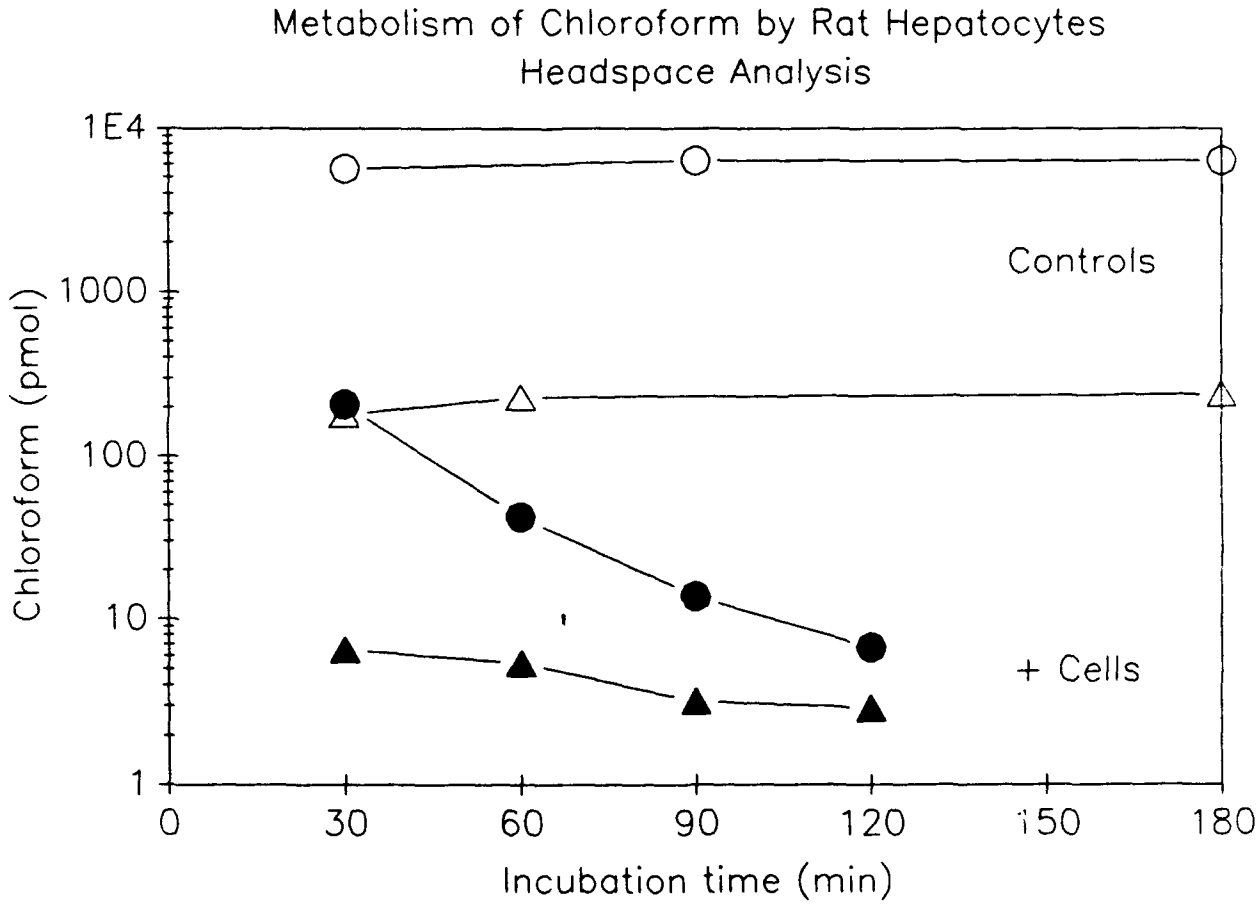

Figure 2. **Metabolism of chloroform by rat hepatocytes--headspace analysis. Rat hepatocytes (4×10^6 cells/ml) were incubated with chloroform, 2400 nmol/flask (circles) and 9350 nmol/flask (triangles) for up to 180 min, with sampling every 30 min. Chloroform in the headspace was determined by gas chromatography. Chloroform contrations in the media were also determined but showed essentially no change (data not shown).**

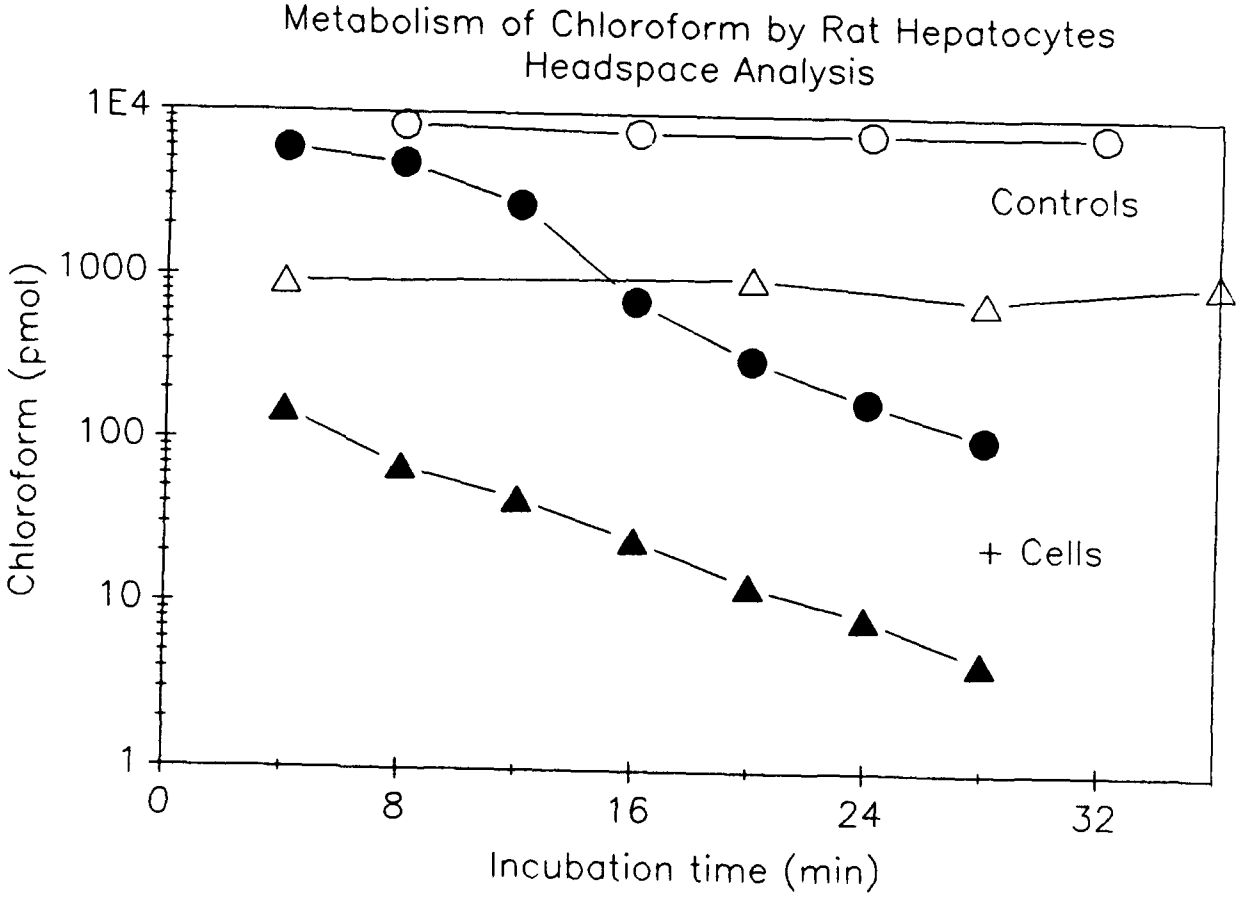

Figure 3. **Metabolism of chloroform by rat hepatocytes--headspace analysis. Rat hepatocytes (4×10^6 cells/ml) were incubated with chloroform, 2400 nmol/flask (circles) and 9350 nmol/flask (triangles) up to 60 min with frequent sampling. Chloroform in the headspace was determined by gas chromatography.**

FUTURE PLANS

The following experiments are planned for the second year of the project:

- The rat hepatocyte experiments with chloroform will be continued. Heat-killed hepatocytes will be included as the control, and the experiments will determine fully the characteristics of the loss of chloroform from both the media and headspace at several different test concentrations. Kinetic constants will be calculated.

- Precision-cut rat liver slices will be compared to hepatocytes as a model system, with chloroform used as a substrate. The chloroform content of the headspace and medium and the total chloroform in the system will be quantitatively determined and the kinetic constants calculated.

- Because human and rat preparations may behave differently in terms of viability and metabolic activity in different *in vitro* systems, the same experiments will be performed with human liver tissue to compare the metabolism of chloroform by human hepatocyte suspensions and human liver slices. If the incubations can be performed in about 30 min, as the data obtained to date suggest, then changes in human hepatocyte viability may not be a significant factor.

- With the *in vitro* system selected from the above experiments, rat liver preparations will be used to determine the kinetic constants for metabolism of the other five halogenated solvents originally proposed--carbon tetrachloride, trichloroethylene, dichloromethane, 1,1,1-trichlrorethane, and bromochloromethane. The values obtained will be compared to published values determined *in vivo*. Modifications of the incubation conditions will be incorporated as needed to obtain a good correlation between the *in vivo* and *in vitro* results.

Much of the work originally planned for the third year of the project---work on the detailed characterization of metabolism of trichloroethylene---has already been completed (Knadle et al., 1990). Thus time is available for the comparison of hepatocytes and precision-cut liver slices, necessary experiments for determining the best *in vitro* preparation for metabolism studies with halogenated hydrocarbons. The determination of kinetic constants for the metabolism of the model compounds by human and mouse liver, therefore, will be continued into the third year of the project.

REFERENCES

Elcombe, C. R. Species differences in carcinogenicity and peroxisome proliferation due to trichloroethylene: A biochemical human hazard assessment. Arch's Toxicol Suppl. **8**, 6-17 (1985).

Gargas, M. L., M. E. Andersen, and H. J. Clewell III. A physiologically based simulation approach for determining metabolic constants from gas uptake data. Toxicol. Appl. Pharmacol. **86**, 341-352 (1986).

Green, C. E., J. E. Dabbs, and C. A. Tyson. Functional integrity of isolated rat hepatocytes prepared by whole liver vs. biopsy perfusion. Anal. Biochem. **129**, 269-276 (1983).

Knadle, S. A., C. E. Green, M. Baugh, M. Vidensek, S. M. Short, X. Partos, and C. A. Tyson. Trichloroethylene biotransformation in human and rat primary hepatocytes. Toxicol. in Vitro **4**, 537-541 (1990).

Krumdieck, C. L., J. E. Dos Santos, and K.-J. Ho. A new instrument for the rapid preparation of tissue slices. Anal. Biochem. **104**, 118-123 (1980).

Pryor, G., C. Rebert, K. Kassay, H. Kuiper, and R. Gordon. The hearing loss associated with exposure to toluene is not caused by a metabolite. Brain Res. Bull. **27**, 109-113, 1991.

Sato, A. and T. Nakajima. A vial-equilibration method to evaluate the drug-metabolizing enzyme activity for volatile hydrocarbons. Toxicol. Appl. Pharmacol. **47**, 41-46 (1979).

Smith, P. F., G. Krack, R. L. McKee, D. G. Johnson, A. J. Gandolfi, V. J. Hruby, C. L. Krumdieck, and K. Brendel. Maintenance of adult rat liver slices in dynamic organ culture. In Vitro Cell. Dev. Biol. **22**, 706-712 (1986).

Tyson, C. A. and C. E. Green. Cytotoxicity Measures: Choices and Methods. In *The Isolated Hepatocyte: Use in Toxicology and Xenobiotic Biotransformations*. E. Rauckman and G. Padilla (Eds.), Academic Press Inc., pp. 119-158, 1987.

Tyson, C. A., K. Hawk-Prather, D. L. Story, and D. H. Gould. Correlations of *in vitro* and *in vivo* hepatotoxicity for five haloalkanes. Toxicol. Appl. Pharmacol. **70**, 289-302 (1983).